# REPORT

## FRESH WATER ALGAL GROWTH INHIBITION TEST

## WITH

[redacted]

NOTOX Project 338783
NOTOX Substance 111834/B

CONFIDENTIALITY STATEMENT

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STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director
Ing. M.H.J. Migchielsen

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Date: November 13, 2002

Management:
Ing. E.J. van de Waart M.Sc.
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.....

Date: 15/11/2002

## QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below.
During the on-site inspections procedures applicable to this type of study were inspected.

| DATES OF QAU INSPECTIONS/AUDITS | REPORTING DATES |
|---|---|
| on-site inspection(s) (Process) | |
| July 08 to 15, 2002 (Ecotoxicology)<br>August 19 to 30, 2002 (Analytical support) | July 17, 2002<br>September 02, 2002 |
| protocol inspection(s) (Study) | |
| July 04, 2002 | July 04, 2002 |
| report audit(s) (Study) | |
| November 05 to 06, 2002 | November 06, 2002 |

Head of Quality Assurance — C.J. Mitchell B.Sc.

Date: 18-Nov-02

## SUMMARY

*Selenastrum capricornutum*, Fresh Water Algal Growth Inhibition Test with [redacted]

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted June 7, 1984; and ISO Standard 8692, First edition, 15 November 1989.

The batch of [redacted] tested was a clear and colourless liquid consisting of two main components, i.e. 28.6% peroxidic compounds and 67% Dimethyl phtalate. [redacted] was completely miscible with test medium at the concentrations tested.

The project started with a range-finding test exposing exponentially growing algal cultures to nomina[redacted]concentrations of 0.1, 1.0, 10 and 100 mg/l. The results showed that the $EC_{50}$ for cell growth inhibition and growth rate reduction was between nominally 1 and 10 mg/l.

The range-finding test was followed by a final $EC_{50}$ test exposing exponentially growing algal cultures to nominal [redacted]concentrations of 0.46, 1.0, 2.2, 4.6 and 10 mg/l. The study further included a blank-control. The initial cell density was $10^4$ cells/ml. The total test period was 72 hours. Samples for determination of actual exposure concentrations were taken from all concentrations at the start, after 24 hours and at the end of the test period.

Analysis of the samples taken during the final test showed that concentrations decreased by more than 20% during the test period. Consequently, Time Weight Average (TWA) exposure concentrations were calculated based on both main components measured.
As the TWA concentrations of the components did only marginally differ, it was acceptable to base the actual test range on the mean of both TWA concentrations. The actual test concentrations were 0.2, 0.5, 1.2 and 6.0 mg/l at nominally 1.0, 2.2, 4.6 and 10 mg/l, respectively.
It was known that the analytical method was not sensitive enough to measure the nominal concentration of 0.46 mg/l. Consequently, this concentration was not measured.

In the controls, cell density increased by an average factor of > 16 within 3 days. Further all test conditions (pH and temperature) remained within the ranges prescribed by the protocol.

[redacted] reduced growth rate[1] of this fresh water algae species significantly at nominally 4.6 mg/l and higher, corresponding to a TWA concentration of 1.2 mg/l.

Toxicity parameters based on TWA exposure concentrations were as follows:

The $EC_{50}$ for cell growth inhibition ($E_BC_{50}$: 0-72h) was 0.98 mg/l with a 95% confidence interval ranging from 0.30 to 3.2 mg/l.
The $EC_{10}$ for cell growth inhibition ($E_BC_{10}$: 0-72h) was 0.28 mg/l with a 95% confidence interval ranging from 0.08 to 0.93 mg/l.

The $EC_{50}$ for growth rate reduction ($E_RC_{50}$: 0-72h) was 1.7 mg/l with a 95% confidence interval ranging from 1.2 to 2.5 mg/l.
The $EC_{10}$ for growth rate reduction ($E_RC_{10}$: 0-72h) was 0.61 mg/l with a 95% confidence interval ranging from 0.42 to 0.90 mg/l.

[1] Since growth rate is derived from the slope under the growth curve in a logarithmic plot, the measure of the specific growth rate is preferable over biomass following from the mathematical nature of exponential growth.

The NOEC for cell growth inhibition based on nominal concentrations was 1.0 mg/l, corresponding to a TWA concentration of 0.2 mg/l.
The NOEC for growth rate reduction based on nominal concentrations was 2.2 mg/l, corresponding to a TWA concentration of 0.5 mg/l.

## PREFACE

| | |
|---|---|
| Sponsor | [redacted] |
| Study Monitor | Dr. C.L.J. Braun<br>SHERA, Regulatory Affairs |
| Testing Facility | NOTOX B.V.<br>Hambakenwetering 7<br>5231 DD 's-Hertogenbosch<br>The Netherlands |
| Aquatic Toxicology:<br>Study Director<br>Technical Coordinator<br>Analytical Chemistry:<br>Principal Scientist | <br>Ing. M.H.J. Migchielsen<br>Mrs. W. Koolen<br><br>Dr. Ir. E. Baltussen |
| Study Plan | Start Project: July 04, 2002<br>Start of first exposure: July 29, 2002<br>Completion last exposure: September 19, 2002<br>Completion Analysis: October 04, 2002<br>Draft report: November 06, 2002<br>Completion project: November 13, 2002 |

## TEST SUBSTANCE

| | |
|---|---|
| Identification<br>Chemical name<br>CAS RN | [redacted] |
| Description | Clear colourless liquid |
| Batch | 1510-14 |
| Purity | See Certificate of Analysis |
| Test substance storage | In refrigerator in the dark |
| Stability under storage conditions | Stable |
| Expiry date | 01 January 2003 |
| Density | Approx. 1160 $kg.m^{-3}$ |
| Stability in water | Unknown |

The sponsor is responsible for all test substance data unless determined by NOTOX.

## PURPOSE

The purpose of the study was to evaluate the test substance for its ability to inhibit the growth of fresh water algae in a short-term experiment.

## GUIDELINES

The study procedures described in this report were based on the ISO International Standard 8692: "Water quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*", First edition, 15 November 1989.

In addition, the procedures were designed to meet the test methods and validity criteria prescribed by the following guidelines:
- European Economic Community (EEC), EEC Directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, C-3: "Algal Inhibition Test" adopted December , 1992.
- Organization for Economic Co-operation and Development (OECD), OECD guideline for Testing of Chemicals, guideline No. 201: "Algae, Growth Inhibition Test", Adopted June 7, 1984.

## ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test substance reference sample and raw data. No data will be withdrawn without the sponsor's written consent.

## DEFINITIONS

- Cell density is the number of cells per millilitre.
- Growth is the increase in cell density over the test period.
- Growth rate is the increase in cell density per unit time. It is derived from the slope under the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass. The $E_RC_{50}$ is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.
- Total growth or biomass is defined as the increase in total cell density over the test period. It is derived from the area under the growth curve in a linear plot. The $E_BC_{50}$ is the concentration of test substance that results in a 50% inhibition of total cell growth relative to the control.
- No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no statistically significant effect on algal growth relative to control values.

## TEST SYSTEM

| | |
|---|---|
| Species | *Selenastrum capricornutum*, strain: NIVA CHL 1. |
| Reason for selection | This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species. |
| Control of sensitivity | The results of the most recent reference test with potassium dichromate (Merck, Art. 4864) are appended to this report. |

## FRESH WATER ALGAE CULTURE

| | |
|---|---|
| Stock culture | Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light (4000-9000 lux) in a climate room at a temperature of 23 ± 2°C. |
| Pre-culture | 3 days before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of $2.10^4$ cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use. |

## PREPARATION OF TEST SOLUTIONS

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system were prevented as much as possible (e.g. film of the test substance on the water surface).

The batch of [redacted] tested was a clear and colourless liquid consisting of two main components, i.e. 28.6% peroxidic compounds and 67% Dimethyl phtalate (see also attached analysis certificate). [redacted] was completely miscible with test medium at the concentrations tested.

Preparation of test solutions started with stock solutions at nominally 100 mg/l (range-finding test) or 10 mg/l (final test). These solutions were magnetically stirred for ca. 20 minutes and additionally treated with ultrasonic waves for 5 minutes during the range-finding test. The resulting, clear and colourless, stock solutions were then used to prepare the lower test concentrations by subsequent dilutions in test medium. After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, adequate volumes of an algal suspension were added to each replicate providing a cell density of $10^4$ cells/ml.

## RANGE-FINDING TEST

A range-finding test preceded the final test to provide information about the range of concentrations to be used in the final test. Algae were exposed to a range of 0.1 to 100 mg/l, increasing by a factor 10. Test procedure and conditions were comparable to those applied in the final test.

## FINAL TEST:

## TEST CONCENTRATIONS

| | |
|---|---|
| TRIGONOX R-938 | 0.46, 1.0, 2.2, 4.6 and 10 mg/l. |
| Controls | Test medium without test substance or other additives (blank). |

| | |
|---|---|
| Replicates | 3 replicates of each test concentration.<br>6 replicates of the blank-control.<br>2 replicates of the highest concentration without algae.<br>1 extra replicate of each test concentration with algae for sampling after 24 hours of exposure. |

## TEST PROCEDURE AND CONDITIONS

| | |
|---|---|
| Test type | Static |
| Test vessels | 100 ml, all-glass |
| Milli-Q water | Tap water purified by reverse osmosis and then passed over activated carbon and ion-exchange cartridges (Millipore Corp., Bedford, Mass., USA). |
| Medium | M2-medium according to the ISO-Standard "Algal growth inhibition test" Nov. 1989; formulated using Milli-Q water preventing precipitation and with the following composition: |

| | | |
|---|---|---|
| $NH_4Cl$ | 15 | mg/l |
| $MgCl_2.6H_2O$ | 12 | mg/l |
| $CaCl_2.2H_2O$ | 18 | mg/l |
| $MgSO_4.7H_2O$ | 15 | mg/l |
| $KH_2PO_4$ | 1.6 | mg/l |
| $FeCl_3.6H_2O$ | 80 | µg/l |
| $Na_2EDTA.2H_2O$ | 100 | µg/l |
| $H_3BO_3$ | 185 | µg/l |
| $MnCl_2.4H_2O$ | 415 | µg/l |
| $ZnCl_2$ | 3 | µg/l |
| $CoCl_2.6H_2O$ | 1.5 | µg/l |
| $CuCl_2.2H_2O$ | 0.01 | µg/l |
| $Na_2MoO_4.2H_2O$ | 7 | µg/l |
| $NaHCO_3$ | 50 | mg/l |
| Hardness (Ca+Mg) | 0.24 | mmol/l (24 mg $CaCO_3$/l) |

| | |
|---|---|
| Cell density | An initial cell density of $1 \times 10^4$ cells/ml. |
| Test duration | 72 hours |
| Illumination | Continuously using TLD-lamps of the type 'Cool-white' of 30 Watt, with a light intensity within the range of 70 to 98 $\mu E.m^{-2}.s^{-1}$, not varying by more than 20%. |
| Incubation | During incubation the algal cells were kept in suspension by continuous shaking. |

## SAMPLING FOR ANALYSIS OF TEST CONCENTRATIONS

During the final test samples for analysis were taken in duplicate from all test concentrations and the blank-control.

| | |
|---|---|
| Frequency | at t=0 h, t=24 h and t=72 h |
| Volume | 6 ml |
| Storage | Samples were stored in a deep-freeze until analysis. |

Compliance with the Quality criteria regarding maintenance of actual concentrations was demonstrated by running a test vessel at the highest substance concentration but without algae and samples for analysis were taken at the start, after 24 hours of exposure and at the end of the test period.

Additionally, reserve samples of 12 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for possible analysis until delivery of the final report with a maximum of three months. The method of analysis is described in the appended Analytical Report.

## MEASUREMENTS

| | |
|---|---|
| pH | At the beginning and at the end of the test.<br>The pH of the solutions should preferably not deviate by more than 1.5 units during the test. |
| Temperature of medium | Every day in a temperature-control vessel. |

## RECORDING OF CELL DENSITIES

At the beginning of the test, cells were counted by microscope, using a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Varian Cary 50 single beam spectrophotometer with immersion probe (pathlength =20 mm). Varian Nederland BV., Houten, The Netherlands. Algal medium was used as blank.

## DATA HANDLING

**Calibration curve:**

Quantification of cell densities was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The equation of this curve was then used to calculate the cell densities of the various test solutions at different points in time during the test period.

**Comparison of areas under the growth curves:**

The area below the growth curve was calculated using the formula:

$$A = \frac{N_1 - N_0}{2} * t_1 + \frac{N_1 + N_2 - 2 * N_0}{2} * (t_2 - t_1) + \frac{N_{n-1} + N_n - 2 * N_0}{2} * (t_n - t_{n-1})$$

Where: A = area
$N_0$= nominal number of cells/ml at the start of the test
$N_1$= measured number of cells/ml at $t_1$
$N_n$= measured number of cells/ml at $t_n$
$t_1$= time of first measurements after beginning of the test
$t_n$= time of $n^{th}$ measurement after beginning of the test

The percentage inhibition of cell growth at each test concentration ($I_T$) was calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} * 100$$

Where: $A_C$ = area below the growth curve obtained in the control
$A_T$ = area below the growth curve at each test substance concentration

Growth inhibition was calculated for the total period of 72h.

**Comparison of growth rates:**

The average specific growth rate ($\mu$) for exponentially growing cultures was calculated as:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

The average growth rate at each test substance concentration was then compared to the control value and the percentage reduction in growth rate was calculated.

**Determination of the average exposure concentrations:**

The Time Weight Average exposure (TWA) concentrations were calculated as:

$$\frac{((C_{t=0} * C_{t=24})^{½} * 24 + (C_{t=24} * C_{t=72})^{½} * 48)}{72}$$

Being the geometric means of the concentrations of [redacted] measured in the samples taken at the start ($C_{t=0}$), after 24 hours ($C_{t=24}$) and the end of the test ($C_{t=72}$).

The final exposure concentration(s) will be taken as a factor of 10 below the limit of detection in case the substance is not detected (i.e. no response above the noise level).
When the test substance is detected but cannot be quantified (i.e. response is just below detection level or below the lower limit of the validated calibration curve), the final exposure concentration will be taken as half the lower limit of quantification (based on the OECD "Guidance document on aquatic toxicity testing of difficult substances and mixtures").

In other cases the concentration will be determined by extrapolation of the calibration curve applied in the analysis of the samples providing that the response is within the calibration curve produced during validation of the analytical method.

**Determination of the NOEC and calculation of the $EC_{50}$:**

For determination of the NOEC and the $EC_{50}$ the approaches recommended in the OECD guideline (201, adopted 7 June 1984) were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth or inhibition of growth rate (ANOVA, Tukey test, Bonferroni t-test, TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Additionally, the $EC_{10}$ was determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993.
Calculation of the $EC_{50}$ and $EC_{10}$ values was based on linear regression analysis of the percentages of growth inhibition and the percentages of growth rate reduction versus the logarithms of the corresponding TWA exposure concentrations of the test substance.

## RESULTS

Range-finding test:

The mean cell densities measured during the range-finding test are presented in Table 1. Table 2 presents the percentages growth inhibition and growth rate reduction per concentration. The results showed that the $EC_{50}$ for cell growth inhibition and growth rate reduction was between nominally 1 and 10 mg/l. Analytical results obtained during the range-finding test with fish (NOTOX Project 338761) showed that [redacted] was not stable in test medium. Consequently, it was decided to analyse all test concentrations at three time-points during the final test.

*Table 1: Mean cell densities ($x10^4$ cells/ml) during the range-finding test*

| Nominal conc. [redacted] | Exposure time (hours) | | | |
|---|---|---|---|---|
| (mg/l) | 0 | 24 | 48 | 72 |
| Blank-control | 1.0 | 4.2 | 24.8 | 100.2 |
| 0.1 | 1.0 | 4.8 | 26.6 | 95.0 |
| 1 | 1.0 | 3.3 | 21.6 | 81.9 |
| 10 | 1.0 | 1.6 | 1.9 | 1.0 |
| 100 | 1.0 | 1.4 | 1.1 | 1.0 |

*Table 2: Percentage reduction of growth rate and inhibition of total growth during the range-finding test*

| Nominal conc. [redacted] | Cell growth (0-72 hrs) | | Mean growth rate | |
|---|---|---|---|---|
| (mg/l) | Mean area (A) | Inhibition (%) | $\mu$ (0-72 hrs) | Reduction (%) |
| Blank-control | 1838.80 | | 0.06391 | |
| 0.1 | 1832.40 | 0.3 | 0.06316 | 1.2 |
| 1 | 1520.00 | 17.3 | 0.06115 | 4.3 |
| 10 | 35.20 | 98.1 | 0.00000 | 100.0 |
| 100 | 12.00 | 99.3 | 0.00000 | 100.0 |

Final test:

Measured test substance concentrations

The results of analysis of the samples taken during the study are described in Tables 1 and 2 of the appended Analytical Report.

An overview of the analytical results including the Time Weight Average (TWA) exposure concentrations, based on both main components, is presented in Table 3 below. TWA concentrations were calculated as a consequence of the fact that concentrations decreased by more than 20% during the test period. For the calculation method see section 'Determination of the average exposure concentrations'.

It was known that the analytical method was not sensitive enough to measure the nominal concentration of 0.46 mg/l. Consequently, this concentration was not measured. The results of analysis also indicated that the measured concentration at nominally 1.0 mg/l was below the limit of detection at all measuring times. At 2.2 and 4.6 mg/l the measured concentration decreased below the detection limit after 72 hours of exposure. In conclusion: actual concentrations at the lower test concentrations were difficult to obtain.
The actual range of [redacted] concentrations tested was based on the average exposure concentrations of both main components. As the TWA concentrations of the components did only marginally differ, it was acceptable to base the actual test range on the mean of both TWA concentrations. The actual test concentrations were 0.2, 0.5, 1.2 and 6.0 mg/l at nominally 1.0, 2.2, 4.6 and 10 mg/l, respectively.

*Table 3: Measured concentrations versus nominal concentrations:*

| **Nominal conc. [redacted] (mg/l)** | **Measured conc. t=0 (mg/l) Peak 1 / Peak 2** | **Measured conc. t=24 (mg/l) Peak 1 / Peak 2** | **Measured conc. t=72 (mg/l) Peak 1 / Peak 2** | **Time Weight Average concentration (mg/l) Peak 1 / Peak 2 / Mean** |
|---|---|---|---|---|
| 1.0 | 0.32[1] / 0.32[1] | 0.32[1] / 0.32[1] | 0.064[2] / 0.063[2] | 0.20 / 0.20 0.2 |
| 2.2 | 1.45 / 1.33 | 0.70 / 0.73 | 0.064[2] / 0.063[2] | 0.48 / 0.47 0.5 |
| 4.6 | 3.27 / 3.08 | 2.55 / 2.40 | 0.064[2] / 0.063[2] | 1.23 / 1.17 1.2 |
| 10 | 8.05 / 7.66 | 7.42 / 6.67 | 4.39 / 3.47 | 6.38 / 5.59 6.0 |
| 10 (w.a.) | 8.45 / 8.00 | 7.98 / 7.36 | 6.42 / 5.51 | 7.51 / 6.80 7.2 |

w.a. without algae

[1] The final exposure concentration was taken as half the lower limit of quantification.

[2] The final exposure concentration was taken as a factor of 10 below the limit of detection as the substance was not detected.

Mean cell densities

Table 4 shows mean cell densities measured at 24-hour intervals at the different concentrations of [redacted] The respective growth curves are shown in Figure 1 (see the Appendix I for the cell densities per replicate).

*Table 4: Mean cell densities during the final test*

| Concentration (mg/l) [redacted] | | Exposure time (hours) | | | |
|---|---|---|---|---|---|
| Nominal | / TWA* | 0 | 24 | 48 | 72 |
| Blank-control | | 1.0 | 3.6 | 31.9 | 127.5 |
| 0.46 | n.m. | 1.0 | 5.5 | 29.2 | 140.2 |
| 1.0 | 0.2 | 1.0 | 5.6 | 31.3 | 132.7 |
| 2.2 | 0.5 | 1.0 | 4.4 | 23.4 | 101.2 |
| 4.6 | 1.2 | 1.0 | 3.8 | 8.1 | 28.9 |
| 10 | 6.0 | 1.0 | 1.4 | 1.8 | 1.0 |

* Time Weight Average exposure concentration.
n.m. not measured.

Inhibition of cell growth and reduction of growth rate

Table 5 shows the calculation of the percentages of inhibition of cell growth and Table 6 the percentages of growth rate reduction at different time intervals (see the Appendix I for the areas of cell growth and values of growth rate per replicate). Statistical analysis of the data for areas under the growth curves (cell growth) and growth rate are shown in Appendix II.

Inhibition of cell growth increased with increasing concentration of [redacted] from nominally 2.2 mg/l upwards resulting in 99% inhibition at nominally 10 mg/l. Statistically significant inhibition of cell growth was found at test concentrations of 2.2 mg/l and higher (Bonferroni-t and Tukey test; $\alpha$ =0.05).

Growth rates were in the range of the controls at the nominal concentrations of 0.46 and 1.0 mg/l during the 72-hour test period, whereas the growth rate of algae exposed to 2.2 mg/l and higher were increasingly reduced.
Statistically significant reduction of growth rate was found at nominal test concentrations of 4.6 mg/l and higher (Bonferroni-t and Tukey test; $\alpha$=0.05).

*Table 5: Percentage inhibition of cell growth during the final test.*

| Concentration (mg/l) [redacted] | | Cell growth (0-72 hrs) | |
|---|---|---|---|
| Nominal | / TWA* | Mean area (A) | Inhibition (%) |
| Blank-control | | 2323.02 | |
| 0.46 | n.m. | 2454.95 | -5.7 |
| 1.0 | 0.2 | 2417.35 | -4.1 |
| 2.2 | 0.5 | 1821.21 | 21.6 |
| 4.6 | 1.2 | 571.28 | 75.4 |
| 10 | 6.0 | 29.41 | 98.7 |

* Time Weight Average exposure concentration.
n.m. not measured.

*Table 6: Percentage reduction of growth rate at different time intervals during the final test.*

| Concentration (mg/l) [redacted] Nominal / TWA* | | Mean growth rate | | | | | |
|---|---|---|---|---|---|---|---|
| | | μ (0-24 hrs) | Reduction (%) | μ (0-48 hrs) | Reduction (%) | μ (0-72 hrs) | Reduction (%) |
| Blank-control | | 0.05327 | | 0.07207 | | 0.06730 | |
| 0.46 | n.m. | 0.07108 | -33.4 | 0.07010 | 2.7 | 0.06861 | -1.9 |
| 1.0 | 0.2 | 0.07138 | -34.0 | 0.07162 | 0.6 | 0.06767 | -0.5 |
| 2.2 | 0.5 | 0.06177 | -16.0 | 0.06560 | 9.0 | 0.06410 | 4.8 |
| 4.6 | 1.2 | 0.05520 | -3.6 | 0.04312 | 40.2 | 0.04595 | 31.7 |
| 10 | 6.0 | 0.01373 | 74.2 | 0.01205 | 83.3 | 0.00000 | 100.0 |

* Time Weight Average exposure concentration.
n.m. not measured.

Experimental conditions

Table 7 shows the pH recorded at the beginning and the end of the test.

The temperature of the test medium was 23.8°C at the start of the test. During the exposure period the temperature measured in the incubator was maintained between 24.1 and 24.7°C.

*Table 7: pH levels recorded during the final study.*

| Concentration (mg/l) [redacted] Nominal / TWA* | | Exposure time (hours) | |
|---|---|---|---|
| | | 0 | 72 |
| Blank-control | | 8.3 | 9.3 |
| 0.46 | n.m. | 8.3 | 9.0 |
| 1.0 | 0.2 | 8.3 | 9.1 |
| 2.2 | 0.5 | 8.3 | 9.0 |
| 4.6 | 1.2 | 8.3 | 8.1 |
| 10 | 6.0 | 8.3 | 7.9 |

* Time Weight Average exposure concentration.
n.m. not measured.

## ACCEPTABILITY OF THE TEST

1. In the controls, cell density increased by an average factor of > 16 within three days.
2. Analysis of samples taken from the solutions with and without algae showed that the actual exposure concentration was not maintained above 80% relative to the initial concentration. This could not be prevented, as the experimental design does not allow for intermittent renewal. It was concluded that the decrease of test substance could partly be due to absorption to the algal cells. In this case, the integrity of the test was not affected, since the algae were still in contact with the substance: it did not leave the system. Alternatively and most likely, the test substance may have been degraded. In this case, the integrity of the study can be considered not affected either, based on the fact that such degradation would occur in every situation in which algae are exposed to an aqueous solution of the test substance. The toxicity observed is then the result of exposure to parent compound and possibly degradation products, which will also occur in the aquatic environment after a possible discharge or spill.
3. Further, all test conditions (pH and temperature) remained within the ranges prescribed by the protocol.

## DETERMINATION OF EC-VALUES

Figures 2 and 3 show the curves for growth inhibition and growth rate reduction versus the log of the concentration. The EC-values with respective 95% confidence intervals have been calculated from these curves (Tables 8 and 9).

## CONCLUSION

Under the conditions of the present study with *Selenastrum capricornutum*, [redacted] reduced growth rate of this fresh water algae species significantly at nominally 4.6 mg/l and higher, corresponding to a TWA concentration of 1.2 mg/l.

Toxicity parameters based on TWA exposure concentrations were as follows:

The $EC_{50}$ for cell growth inhibition ($E_BC_{50}$: 0-72h) was 0.98 mg/l with a 95% confidence interval ranging from 0.30 to 3.2 mg/l.
The $EC_{10}$ for cell growth inhibition ($E_BC_{10}$: 0-72h) was 0.28 mg/l with a 95% confidence interval ranging from 0.08 to 0.93 mg/l.

As growth rate is derived from the slope under the growth curve in a logarithmic plot, the measure of the specific growth rate is preferable over biomass following from the mathematical nature of exponential growth.

The $EC_{50}$ for growth rate reduction ($E_RC_{50}$: 0-72h) was 1.7 mg/l with a 95% confidence interval ranging from 1.2 to 2.5 mg/l.
The $EC_{10}$ for growth rate reduction ($E_RC_{10}$: 0-72h) was 0.61 mg/l with a 95% confidence interval ranging from 0.42 to 0.90 mg/l.

The NOEC for cell growth inhibition based on nominal concentrations was 1.0 mg/l, corresponding to a TWA concentration of 0.2 mg/l.
The NOEC for growth rate reduction based on nominal concentrations was 2.2 mg/l, corresponding to a TWA concentration of 0.5 mg/l.

*Figure 1: Growth curves at different concentrations of [redacted]*

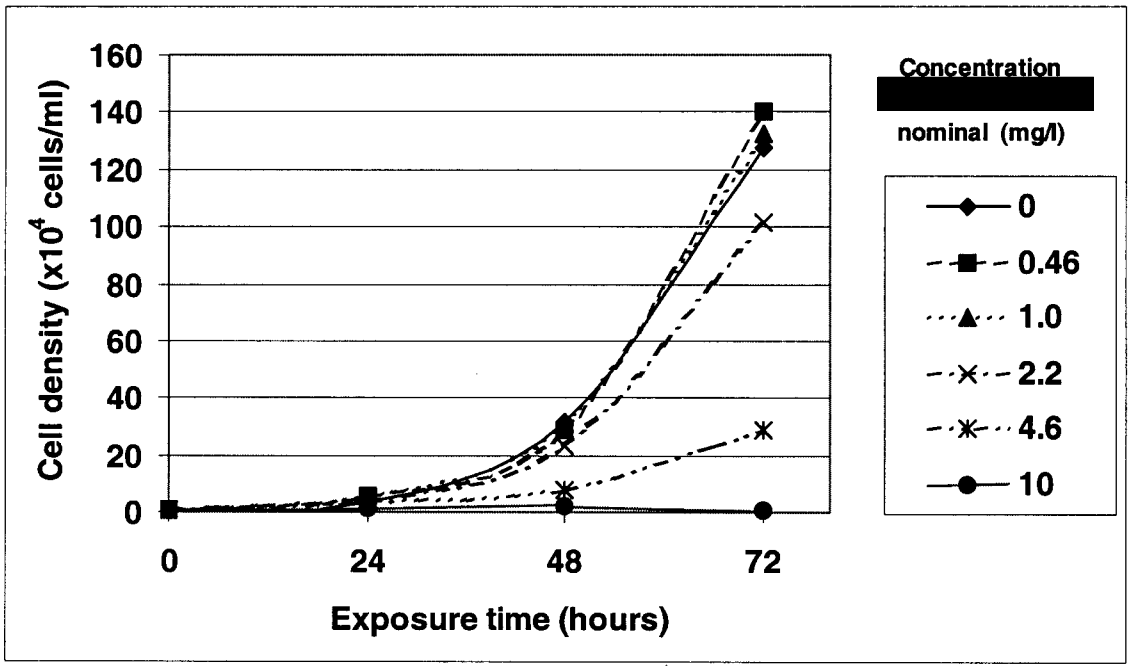


*Table 8: EC-values for growth inhibition:*

| Concentration | X | Y |
|---|---|---|
| TWA (mg/l) | Log conc. (mg/l) | Inhibition (%) |
| 0.2 | -0.699 | 2.5 |
| 0.2 | -0.699 | 12.1 |
| 0.2 | -0.699 | -26.8 |
| 0.5 | -0.301 | 27.1 |
| 0.5 | -0.301 | 23.0 |
| 0.5 | -0.301 | 14.7 |
| 1.2 | 0.079 | 79.0 |
| 1.2 | 0.079 | 81.8 |
| 1.2 | 0.079 | 65.4 |
| 6 | 0.778 | 98.0 |
| 6 | 0.778 | 99.1 |
| 6 | 0.778 | 99.1 |

| | |
|---|---|
| Slope: | 72.4974 |
| Intercept: | 50.5063 |
| Multiple R: | 0.9380 |
| n = number of observations: | 12 |

Regression line: Y= 72.50 X + 50.51

| Prediction of X values based on known Y values | | | |
|---|---|---|---|
| Known Y | $10^{Xreg}$ | $10^{X95\%-}$ | $10^{X95\%+}$ |
| Inhibition (%) | (mg/l) | (mg/l) | (mg/l) |
| 10 | 0.28 | 0.08 | 0.93 |
| 25 | 0.44 | 0.14 | 1.46 |
| 50 | 0.98 | 0.30 | 3.19 |
| 100 | 4.82 | 1.38 | 16.86 |

*Figure 2: Percentage inhibition of cell growth as function of the log TWA concentration (mg/l) of [redacted] Dashed curves represent the 95 % confidence limits.*

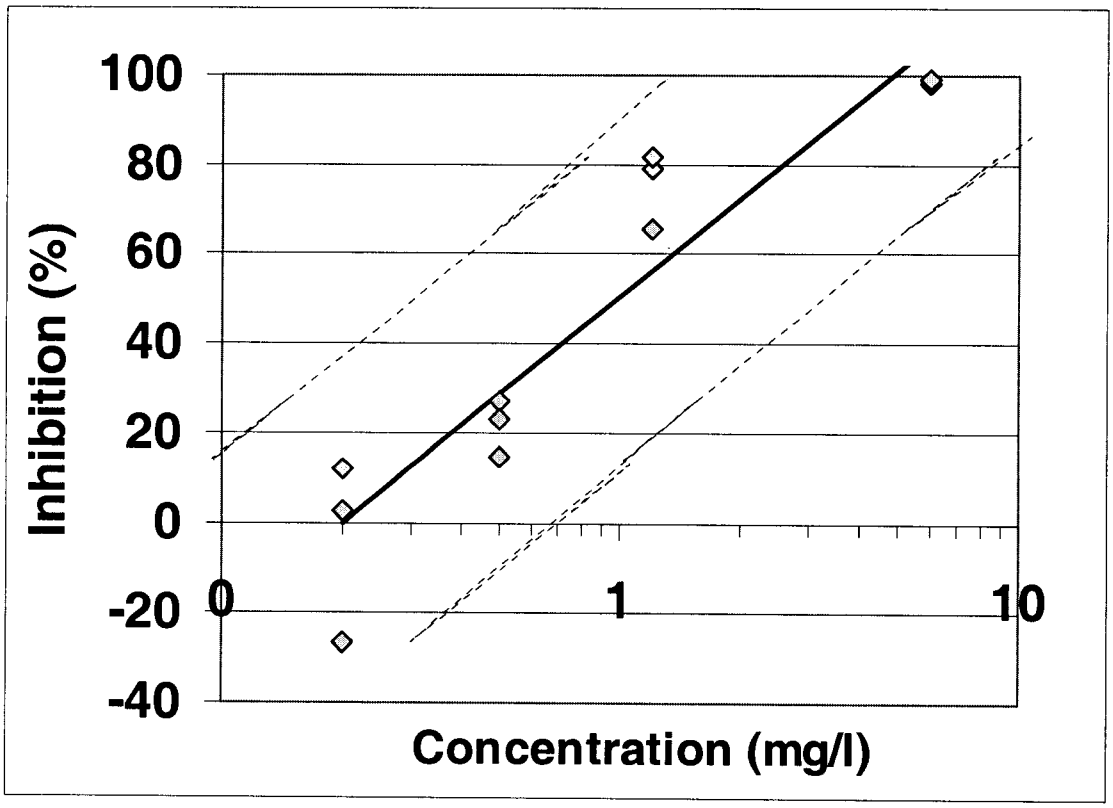


*Table 9: EC-values for growth rate reduction:*

| Concentration | X | Y |
|---|---|---|
| TWA (mg/l) | Log conc. (mg/l) | Reduction (%) |
| 0.2 | 0.00 | 0.94 |
| 0.2 | 0.00 | 3.00 |
| 0.2 | 0.00 | -5.57 |
| 0.5 | -0.30 | 6.34 |
| 0.5 | -0.30 | 4.77 |
| 0.5 | -0.30 | 3.16 |
| 1.2 | 0.08 | 34.23 |
| 1.2 | 0.08 | 38.36 |
| 1.2 | 0.08 | 22.57 |
| 6.0 | 0.78 | 100.00 |
| 6.0 | 0.78 | 100.00 |
| 6.0 | 0.78 | 100.00 |

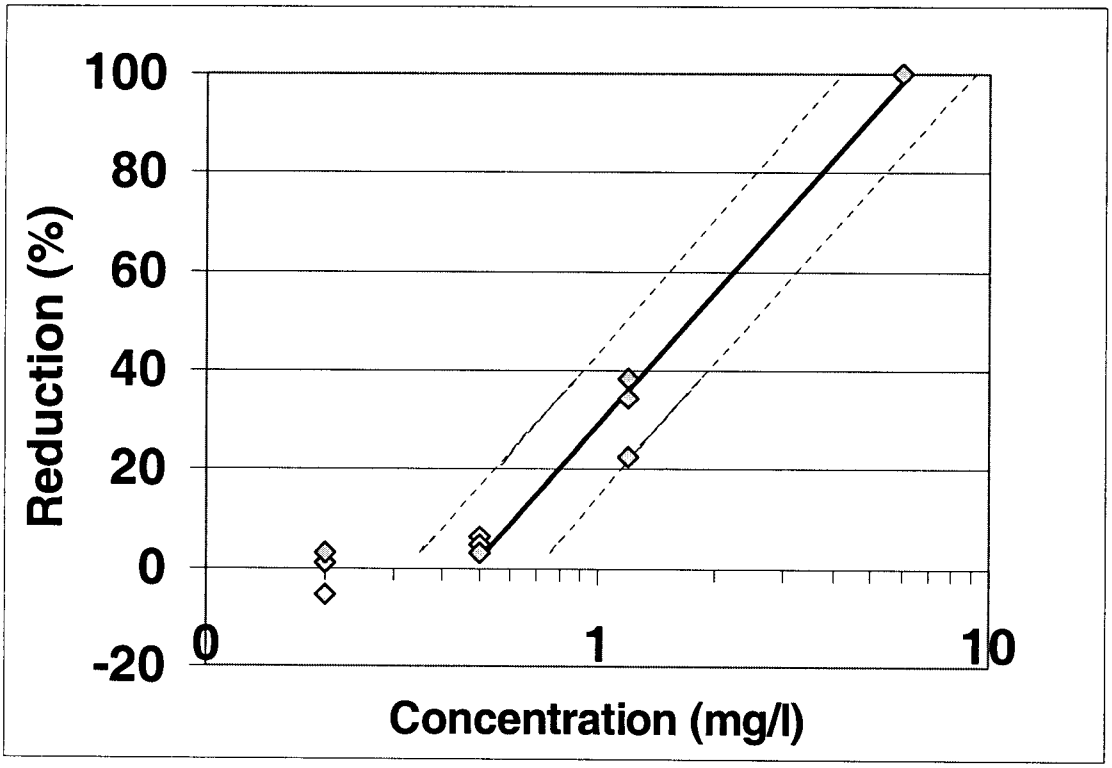
| | |
|---|---|
| Slope: | 89.4242 |
| Intercept: | 28.9110 |
| Multiple R: | 0.9923 |
| n = number of observations: | 9 |

Regression line: Y= 89.42 X + 28.91

Prediction of X values based on known Y values

| Known Y Reduction (%) | $10^{Xreg}$ (mg/l) | $10^{X95\%-}$ (mg/l) | $10^{X95\%+}$ (mg/l) |
|---|---|---|---|
| 10 | 0.61 | 0.42 | 0.90 |
| 25 | 0.90 | 0.63 | 1.31 |
| 50 | 1.72 | 1.20 | 2.47 |
| 100 | 6.24 | 4.20 | 9.26 |

*Figure 3: Percentage reduction of growth rate as function of the log TWA concentration (mg/l) of [redacted] Dashed curves represent the 95 % confidence limits.*



REFERENCE TEST

*Selenastrum capricornutum*, fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 356636).

Start of first exposure: August 06, 2002
Completion last exposure: August 09, 2002

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted June 7, 1984; and ISO Standard 8692, First edition, 15 November 1989.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to POTASSIUM DICHROMATE (Merck, Art. 4864, Batch K28974764).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a blank-control. The initial cell density was $1.0 \times 10^4$ cells/ml.

Results:

Calculation of % reduction in growth rate in the reference test:

| **Concentration $K_2Cr_2O_7$ (mg/l)** | **Growth rate: Interval 0-72h** | **Reduction %** |
|---|---|---|
| Blank-control | 0.05593 | |
| 0.18 | 0.06161 | -10.1 |
| 0.32 | 0.05967 | -6.7 |
| 0.56 | 0.06014 | -7.5 |
| 1.0 | 0.05817 | -4.0 |
| 1.8 | 0.03882 | 30.6 |
| 3.2 | 0.01629 | 70.9 |

Under the conditions of the reference study with *Selenastrum capricornutum*, potassium dichromate reduced growth rate of this fresh water algae species at nominal concentrations of 1.8 mg/l and higher.

The $EC_{50}$ for growth rate reduction ($E_RC_{50}$: 0-72h) was 2.4 mg/l with a 95 % confidence interval ranging from 2.1 to 2.7 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Although the value for the $E_RC_{50}$: 0-72h slightly exceeded this range, the lower limit of the 95 % confidence interval was within this standard range.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

# APPENDIX I

WORKSHEET DATA

*Table I3: Cell densities calculated from the individual extinction values*

| **Number of inoculated cells at t=0:** | | **1 x10$^4$ cells/ml** | | | |
|---|---|---|---|---|---|
| **Nominal conc.** [redacted] **(mg/l)** | **Vessel number** | **Exposure time (hours)** | | | |
| | | **0** | **24** | **48** | **72** |
| Blank control | 1 | 1.00 | 2.92 | 32.62 | 130.38 |
| | 2 | 1.00 | 3.59 | 34.11 | 147.40 |
| | 3 | 1.00 | 3.17 | 32.78 | 125.21 |
| | 4 | 1.00 | 3.55 | 26.40 | 119.00 |
| | 5 | 1.00 | 4.08 | 33.11 | 120.57 |
| | 6 | 1.00 | 4.46 | 32.41 | 122.56 |
| 0.46 | 1 | 1.00 | 5.04 | 24.79 | 132.33 |
| | 2 | 1.00 | 5.57 | 28.39 | 155.06 |
| | 3 | 1.00 | 5.95 | 34.40 | 133.08 |
| 1.0 | 1 | 1.00 | 5.16 | 30.92 | 121.52 |
| | 2 | 1.00 | 5.41 | 27.23 | 109.97 |
| | 3 | 1.00 | 6.11 | 35.80 | 166.58 |
| 2.2 | 1 | 1.00 | 4.41 | 21.89 | 93.53 |
| | 2 | 1.00 | 4.04 | 22.51 | 100.94 |
| | 3 | 1.00 | 4.79 | 25.70 | 109.14 |
| 4.6 | 1 | 1.00 | 3.38 | 7.31 | 24.21 |
| | 2 | 1.00 | 3.67 | 6.53 | 19.82 |
| | 3 | 1.00 | 4.29 | 10.42 | 42.59 |
| 10 | 1 | 1.00 | 1.47 | 2.47 | 1.00 |
| | 2 | 1.00 | 1.39 | 1.47 | 1.00 |
| | 3 | 1.00 | 1.31 | 1.56 | 1.00 |

# APPENDIX I

WORKSHEET DATA; CONTINUED

*Table I4: Calculation of growth (area under growth curve) and growth rate*

| Nominal conc. [redacted] (mg/l) | Vessel number | Area (A) | Growth rate | | | Growth inhib. (%) | Growth rate reduction (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0-72 hrs | 0-24 hrs | 0-48 hrs | 0-72 hrs | 0-72 hrs | 0-24 hrs | 0-48 hrs | 0-72 hrs |
| Blank control | 1 | 2357.55 | 0.04471 | 0.07260 | 0.06765 | | | | |
| | 2 | 2613.47 | 0.05322 | 0.07353 | 0.06935 | | | | |
| | 3 | 2305.37 | 0.04811 | 0.07271 | 0.06708 | | | | |
| | 4 | 2086.73 | 0.05273 | 0.06820 | 0.06638 | | | | |
| | 5 | 2279.53 | 0.05862 | 0.07291 | 0.06656 | | | | |
| | 6 | 2295.44 | 0.06226 | 0.07247 | 0.06679 | 2323 | 0.05327 | 0.07207 | 0.06730 |
| 0.46 | 1 | 2243.76 | 0.06736 | 0.06688 | 0.06785 | 3 | -26 | 7 | -1 |
| | 2 | 2615.95 | 0.07159 | 0.06971 | 0.07005 | -13 | -34 | 3 | -4 |
| | 3 | 2505.14 | 0.07429 | 0.07371 | 0.06793 | -8 | -39 | -2 | -1 |
| 1.0 | 1 | 2264.13 | 0.06837 | 0.07149 | 0.06667 | 3 | -28 | 1 | 1 |
| | 2 | 2043.00 | 0.07033 | 0.06884 | 0.06528 | 12 | -32 | 4 | 3 |
| | 3 | 2944.91 | 0.07543 | 0.07454 | 0.07105 | -27 | -42 | -3 | -6 |
| 2.2 | 1 | 1693.66 | 0.06187 | 0.06429 | 0.06303 | 27 | -16 | 11 | 6 |
| | 2 | 1788.58 | 0.05820 | 0.06488 | 0.06409 | 23 | -9 | 10 | 5 |
| | 3 | 1981.38 | 0.06525 | 0.06763 | 0.06518 | 15 | -22 | 6 | 3 |
| 4.6 | 1 | 487.14 | 0.05074 | 0.04145 | 0.04426 | 79 | 5 | 42 | 34 |
| | 2 | 422.54 | 0.05417 | 0.03908 | 0.04148 | 82 | -2 | 46 | 38 |
| | 3 | 804.18 | 0.06068 | 0.04883 | 0.05211 | 65 | -14 | 32 | 23 |
| 10 | 1 | 46.64 | 0.01619 | 0.01883 | 0.00000 | 98 | 70 | 74 | 100 |
| | 2 | 20.80 | 0.01378 | 0.00809 | 0.00000 | 99 | 74 | 89 | 100 |
| | 3 | 20.80 | 0.01122 | 0.00923 | 0.00000 | 99 | 79 | 87 | 100 |

# APPENDIX II

## STATISTICS: CELL GROWTH (0-72 HOURS)

**Chi-Square Test for Normality**

Actual and Expected Frequencies

| INTERVAL | <-1.5 | -1.5 to <-0.5 | -0.5 to 0.5 | >0.5 to 1.5 | >1.5 |
|---|---|---|---|---|---|
| EXPECTED | 1.4070 | 5.0820 | 8.0220 | 5.0820 | 1.4070 |
| OBSERVED | 0 | 7 | 8 | 5 | 1 |

Chi-Square = 2.2500 (p-value = 0.6899)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

**Bartlett's Test for Homogeneity of Variance**

Calculated B1 statistic = 11.9000 (p-value = 0.0362)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Critical B = 15.0863 (alpha = 0.01, df = 5)
= 11.0705 (alpha = 0.05, df = 5)

Using Average Degrees of Freedom
(Based on average replicate size of 3.50)

Calculated B2 statistic = 11.2832 (p-value = 0.0460)

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

**ANOVA Table**

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| Between | 5 | 17820433.5988 | 3564086.7197 | 67.9993 |
| Within (Error) | 15 | 786203.9655 | 52413.5977 | |
| Total | 20 | 18606637.5642 | | |

(p-value = 0.0000)

Critical F = 4.5556 (alpha = 0.01, df = 5,15)
= 2.9013 (alpha = 0.05, df = 5,15)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

# APPENDIX II

STATISTICS: CELL GROWTH (0-72 HOURS); CONTINUED

**Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment**

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | t STAT | SIG 0.05 |
|---|---|---|---|---|---|
| 1 | Blank control | 2323.0150 | 2323.0150 | | |
| 2 | 0.46 | 2454.9500 | 2454.9500 | -0.8150 | |
| 3 | 1 | 2417.3467 | 2417.3467 | -0.5827 | |
| 4 | 2.2 | 1821.2067 | 1821.2067 | 3.0998 | * |
| 5 | 4.6 | 571.2867 | 571.2867 | 10.8208 | * |
| 6 | 10 | 29.4133 | 29.4133 | 14.1681 | * |

Bonferroni t critical value = 2.6025 (1 Tailed, alpha = 0.05, df = 5,15)

**Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment**

| GROUP | IDENTIFICATION | NUM OF REPS | MIN SIG DIFF (IN ORIG. UNITS) | % OF CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | Blank control | 6 | | | |
| 2 | 0.46 | 3 | 421.3029 | 18.1 | -131.9350 |
| 3 | 1 | 3 | 421.3029 | 18.1 | -94.3317 |
| 4 | 2.2 | 3 | 421.3029 | 18.1 | 501.8083 |
| 5 | 4.6 | 3 | 421.3029 | 18.1 | 1751.7283 |
| 6 | 10 | 3 | 421.3029 | 18.1 | 2293.6017 |

**Tukey Method of Multiple Comparisons**

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | ORIGINAL MEAN | GROUP 6 | 5 | 4 | 1 | 3 | 2 |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 10 | 29.4133 | 29.4133 | \ | | | | | |
| 5 | 4.6 | 571.2867 | 571.2867 | . | \ | | | | |
| 4 | 2.2 | 1821.2067 | 1821.2067 | * | * | \ | | | |
| 1 | Blank control | 2323.0150 | 2323.0150 | * | * | * | \ | | |
| 3 | 1 | 2417.3467 | 2417.3467 | * | * | * | . | \ | |
| 2 | 0.46 | 2454.9500 | 2454.9500 | * | * | * | . | . | \ |

* = significant difference (alpha = 0.05) . = no significant difference

Tukey critical value = 4.5950 (df = 6,15) s = 52413.5977

# APPENDIX II

## STATISTICS: GROWTH RATE (0-72 HOURS)

**Chi-Square Test for Normality**

Actual and Expected Frequencies

| INTERVAL | <-1.5 | -1.5 to <-0.5 | -0.5 to 0.5 | >0.5 to 1.5 | >1.5 |
|---|---|---|---|---|---|
| EXPECTED | 1.2060 | 4.3560 | 6.8760 | 4.3560 | 1.2060 |
| OBSERVED | 0 | 7 | 6 | 4 | 1 |

Chi-Square = 2.9867 (p-value = 0.5600)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

**Bartlett's Test for Homogeneity of Variance**

Calculated B1 statistic = 10.0769 (p-value = 0.0392)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Critical B = 13.2767 (alpha = 0.01, df = 4)
= 9.4877 (alpha = 0.05, df = 4)

Using Average Degrees of Freedom
(Based on average replicate size of 3.60)

Calculated B2 statistic = 7.3489 (p-value = 0.1186)

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

**ANOVA Table**

| SOURCE | DF | SS | MS | F |
|---|---|---|---|---|
| Between | 4 | 0.0011 | 0.0003 | 41.0773 |
| Within (Error) | 13 | 0.0001 | 0.0000 | |
| Total | 17 | 0.0012 | | |

(p-value = 0.0000)

Critical F = 5.2053 (alpha = 0.01, df = 4,13)
= 3.1791 (alpha = 0.05, df = 4,13)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

# APPENDIX II

STATISTICS: GROWTH RATE (0-72 HOURS); CONTINUED

**Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment**

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | t STAT | SIG 0.05 |
|---|---|---|---|---|---|
| 1 | Blank control | 0.0673 | 0.0673 | | |
| 2 | 0.46 | 0.0686 | 0.0686 | -0.7018 | |
| 3 | 1 | 0.0677 | 0.0677 | -0.1958 | |
| 4 | 2.2 | 0.0641 | 0.0641 | 1.7173 | |
| 5 | 4.6 | 0.0460 | 0.0460 | 11.4526 | * |

Bonferroni t critical value = 2.5326 (1 Tailed, alpha = 0.05, df = 4,13)

**Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment**

| GROUP | IDENTIFICATION | NUM OF REPS | MIN SIG DIFF (IN ORIG. UNITS) | % OF CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | Blank control | 6 | | | |
| 2 | 0.46 | 3 | 0.0047 | 7.0 | -0.0013 |
| 3 | 1 | 3 | 0.0047 | 7.0 | -0.0004 |
| 4 | 2.2 | 3 | 0.0047 | 7.0 | 0.0032 |
| 5 | 4.6 | 3 | 0.0047 | 7.0 | 0.0214 |

**Tukey Method of Multiple Comparisons**

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | ORIGINAL MEAN | GROUP 0 0 0 0 0 5 4 1 3 2 |
|---|---|---|---|---|
| 5 | 4.6 | 0.0460 | 0.0460 | \ |
| 4 | 2.2 | 0.0641 | 0.0641 | * \ |
| 1 | Blank control | 0.0673 | 0.0673 | * . \ |
| 3 | 1 | 0.0677 | 0.0677 | * . . \ |
| 2 | 0.46 | 0.0686 | 0.0686 | * . . . \ |

* = significant difference (alpha = 0.05) . = no significant difference

Tukey critical value = 4.4530 (df = 5,13) s = 0.0000

CERTIFICATE OF ANALYSIS

[redacted]

# *Certificate of Analysis*

[redacted]

TNA-2001007
page 1 of 2

ICS-331

**Product name :** [redacted]
**Chemical name :**
**Batch number : 1510-14**

**Test results:**

| Method | Analysis of | Unit | Result *1 |
|---|---|---|---|
| Jo/72.11, Jo/95.2 | Peroxidic compounds (sum) ***See page 2 for a specification*** | % m/m | 28.6 (± 1.5) |
| J20010792 | [redacted] | % m/m | 67.0 (± 1.0) |
| J20010792 | | % m/m | 2.0 (± 0.3) |
| Amp/88.9 | Water | % m/m | 2.6 (± 0.3) |
| J20010792 | Unidentified impurities | % m/m | 0.5 (± 0.2) |

*1 bracketed values are estimated 95% confidence intervals

File code : **TNA-2001007**
Analytical documentation : 20010792

[redacted]

CERTIFICATE OF ANALYSIS

[redacted]

## *Certificate of Analysis*

[redacted]



[redacted] batch 1510-14: specification of the peroxidic compounds

| structure | % m/m |
|---|---|
| [redacted] | |

[redacted]

# ANALYTICAL REPORT

## FRESH WATER ALGAL GROWTH INHIBITION TEST

## WITH

[redacted]

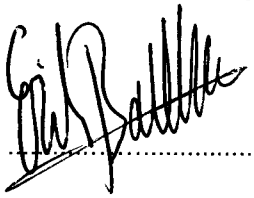
## DETERMINATION OF THE CONCENTRATIONS

**NOTOX Project 338783**
**NOTOX Substance 111834/B**

REPORT APPROVAL

PRINCIPAL SCIENTIST:

Dr. Ir. E. Baltussen
(Analytical Chemistry)



Date: 07 NOV 2002

## PREFACE

| | |
|---|---|
| Study plan<br>(analytical study) | Start: 30 September 2002<br>Completed: 04 October 2002 |

## PURPOSE

The purpose of the analytical study was to determine the test concentrations.

## REAGENTS

| | |
|---|---|
| Acetonitrile | HPLC-grade, Labscan, Dublin, Ireland |
| Milli-Q water | Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore, Bedford, MA, USA |
| M2-medium | Medium formulated according to ISO/IS 8692 |

## SAMPLE PRETREATMENT

All samples were stored in a deep freeze. On the day of analysis, the frozen samples were defrosted at room temperature.

The entire volume of each sample (6 ml) was transferred quantitatively into a 6 ml vial.

## HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC CONDITIONS

Quantitative analyses were based on the area of two peaks (MIPKP-T3 peak 1 and MIPKP-T3 peak 2) with retention times of 13.6 and 14.5 minutes in the HPLC chromatogram of [redacted] (See NOTOX Project 352968: "Implementation and validation of an analytical method for Trigonox R-938").

### Analytical conditions

A SPE-LC method was implemented and validated under Notox Project 352968. This method was based on a Zorbax RX-C18 column using a gradient of acetonitrile and Milli-Q water as the mobile phase, a column temperature of 25°C and a spectrophotometric detector set to read the absorbance at 220 nm.

### Standard and calibration solutions

Standard solutions of [redacted] were prepared in acetonitrile.

Calibration solutions in M2-medium were made up from two standard solutions.

## DATA HANDLING

### General

Mean:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

where
$x_i$ = measured value
$n$ = number of measurements

Maximum deviation: [(highest value - lowest value)/mean] * 100%
where 'mean' is the mean value of the highest and the lowest value.

### Calibration

Response: R = Peak area test substance [units]

Calibration curve: The response was correlated with the concentration test substance, using linear regression analysis (least squares method).

R = a * C + b

R = response calibration solution [units]
C = concentration of test substance in calibration solution [mg/l]
a = slope [units*l/mg]
b = intercept [units]

During analysis, a calibration curve was constructed using six concentrations. For each concentration, two responses were used. The coefficient of correlation was > 0.99.

### Samples

Concentration of [redacted] analysed in the samples:

$$C = \frac{(R-b) * d}{a} \text{ [mg/l]}$$

R = response sample [units]
d = dilution factor
a = slope [units*l/mg]
b = intercept [units]

Limit of detection

Limit of detection:

The limit of detection is defined as the absolute amount of substance at which its signal is three times the noise level.

Limit of detection = ((3 * noise level)/ signal) * conc.

where
noise level = height of the noise [mV]
signal = height of the test substance peak [mV]
conc. = concentration of [redacted] in the test solution [mg/l]

## RESULTS

HPLC chromatograms of a standard solution and of samples from the final test are shown in Figure 1.

Table 1-2 show the analytical results of this study*.

Table 1 Concentrations in test medium based on MIPKP-T3 peak 1 (final test).

| Time of sampling [hours] | Date of sampling [dd-mm-yy] | Date of analysis [2] [dd-mm-yy] | Concentration | | |
|---|---|---|---|---|---|
| | | | Nominal [mg/l] | Analysed [1] [mg/l] | Relative to nominal [%] |
| 0 | 16-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | <0.64 | <64 |
| | | 02-10-02 | 2.2 | 1.45 | 66 |
| | | 04-10-02 | 4.6 | 3.27 | 71 |
| | | 30-09-02 | 10 | 8.05 | 81 |
| | | 30-09-02 | 10 [3] | 8.45 | 84 |
| 24 | 17-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | <0.64 | <64 |
| | | 02-10-02 | 2.2 | 0.696 | 32 [4] |
| | | 04-10-02 | 4.6 | 2.55 | 55 |
| | | 30-09-02 | 10 | 7.42 | 74 |
| | | 30-09-02 | 10 [3] | 7.98 | 80 |
| 72 | 19-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | n.d. | <64 |
| | | 02-10-02 | 2.2 | n.d. | <29 |
| | | 04-10-02 | 4.6 | n.d. | <14 |
| | | 30-09-02 | 10 | 4.39 | 44 |
| | | 30-09-02 | 10 [3] | 6.42 | 64 |

[1] Mean of duplicate samples. The maximum deviation between the responses was calculated for each sample and was < 10%.

[2] Samples were frozen until analysis.

[3] Without algae.

[4] Extrapolated from the calibration curve.

n.d. Not detected. The limit of detection was determined to be 0.64 mg/l based on peak 1.

n.a. Not applicable.

* All recoveries and relative values were calculated using not-rounded concentrations. Therefore, some differences might be observed when calculating the recoveries and relative values using the concentrations as mentioned in the tables.

Table 2 Concentrations in test medium based on MIPKP-T3 peak 2 (final test).

| Time of sampling [hours] | Date of sampling [dd-mm-yy] | Date of analysis [2] [dd-mm-yy] | Concentration | | |
|---|---|---|---|---|---|
| | | | Nominal [mg/l] | Analysed [1] [mg/l] | Relative to nominal [%] |
| 0 | 16-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | <0.63 | <63 |
| | | 02-10-02 | 2.2 | 1.33 | 60 |
| | | 04-10-02 | 4.6 | 3.08 | 67 |
| | | 30-09-02 | 10 | 7.66 | 77 |
| | | 30-09-02 | 10 [3] | 8.00 | 80 |
| 24 | 17-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | <0.63 | <63 |
| | | 02-10-02 | 2.2 | 0.726 | 33 [4] |
| | | 04-10-02 | 4.6 | 2.40 | 52 |
| | | 30-09-02 | 10 | 6.67 | 67 |
| | | 30-09-02 | 10 [3] | 7.36 | 74 |
| 72 | 19-09-02 | 03-10-02 | 0 | n.d. | n.a. |
| | | 01-10-02 | 1 | n.d. [5] | n.a. |
| | | 02-10-02 | 2.2 | n.d. | <29 |
| | | 04-10-02 | 4.6 | n.d. | <14 |
| | | 30-09-02 | 10 | 3.47 | 35 |
| | | 30-09-02 | 10 [3] | 5.51 | 55 |

[1] Mean of duplicate samples. The maximum deviation between the responses was calculated for each sample and was ≤ 12.6%.

[2] Samples were frozen until analysis.

[3] Without algae.

[4] Extrapolated from the calibration curve.

[5] A baseline disturbance at the test substance position was observed in this sample. Based on the peak shape and position of this peak it was concluded that this peak most likely does not correspond to the test substance. The peak area corresponded with a test substance concentration of 0.891 mg/l.

n.d. Not detected. The limit of detection was determined to be 0.63 mg/l based on peak 2.

n.a. Not applicable.

Note:

The test substance is a peroxide which is not very stable in M2-medium at concentration levels below 10 mg/l. Therefore, the actual concentrations might be somewhat higher than mentioned in the tables above, due to decomposition prior to injection of the samples into the HPLC system.

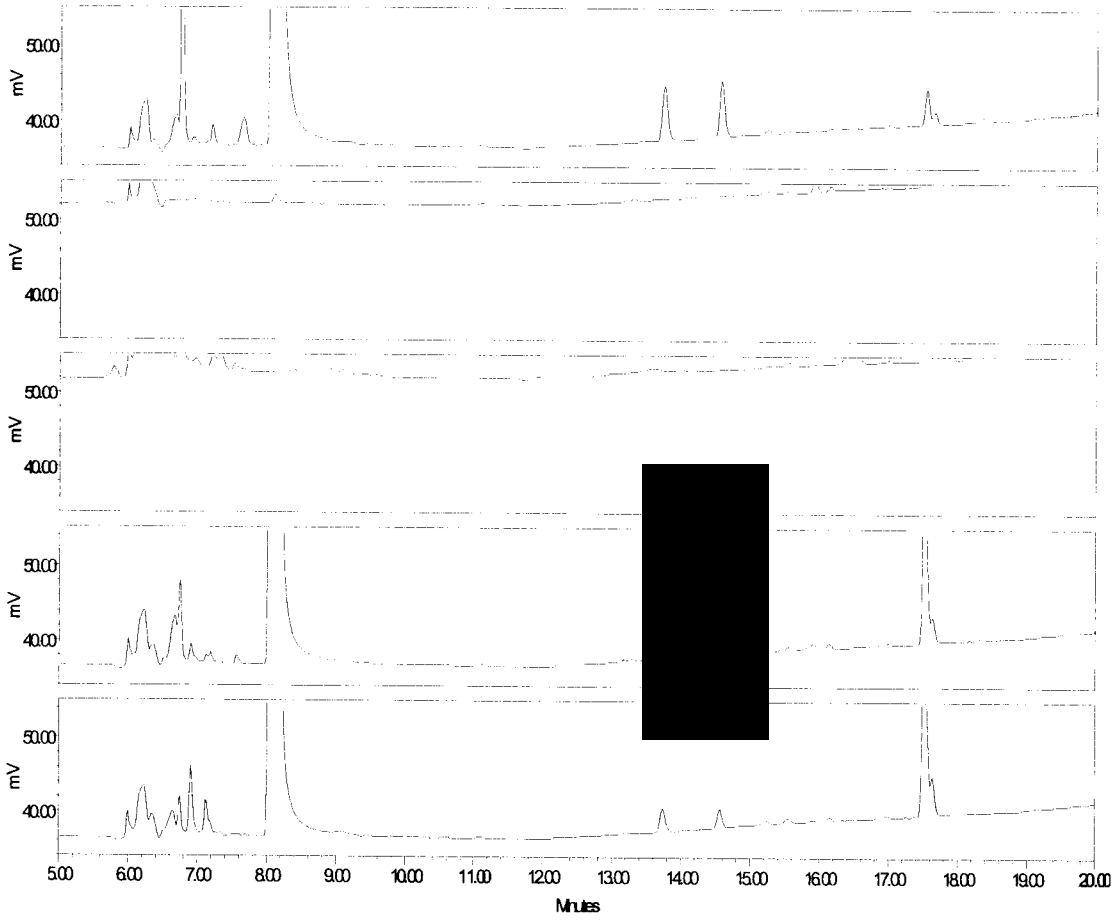



Figure 1 HPLC chromatograms of a standard solution and of samples from the final test. From top to bottom:

- A 9.99 mg/l standard solution in M2-medium [res.id 1157]
- A 0 mg/l nominal concentration sample at t=0 hours [res.id 1447]
- A 0 mg/l nominal concentration sample at t=72 hours [res.id 1450]
- A 100 mg/l nominal concentration sample at t=0 hours [res.id 1194]
- A 100 mg/l nominal concentration sample at t=72 hours [res.id 1202]